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Rapid field detection assays for *Bacillus anthracis*, *Brucella* spp., *Francisella tularensis* and *Yersinia pestis*

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Abstract

Rapid detection is essential for timely initiation of medical post-exposure prophylactic measures in the event of intentional release of biological threat agents. We compared real-time PCR assay performance between the Applied Biosystems 7300/7500 and the RAZOR instruments for specific detection of the causative agents of anthrax, brucellosis, tularemia and plague. Furthermore, an assay detecting *Bacillus thuringiensis*, a *Bacillus anthracis* surrogate, was developed for field-training purposes. Assay sensitivities for *B. anthracis*, *Brucella* spp., *Francisella tularensis* and *Yersinia pestis* were 10–100 fg of target DNA per reaction, and no significant difference in assay performance was observed between the instrument platforms. Specificity testing of the diagnostic panels with both instrument platforms did not reveal any cross-reactivity with other closely related bacteria. The duration of thermocycling with the RAZOR instrument was shorter, i.e. 40 min as compared with 100 min for the Applied Biosystems 7300/7500 instruments. These assays provide rapid tools for the specific detection of four biological threat agents. The detection assays, as well as the training assay for *B. thuringiensis* powder preparation analysis, may be utilized under field conditions and for field training, respectively.

Keywords: *Bacillus anthracis*, *Bacillus thuringiensis*, *Brucella* spp., *Francisella tularensis*, real-time PCR, *Yersinia pestis*
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Introduction

Bacillus anthracis, *Brucella* spp., *Francisella tularensis* and *Yersinia pestis* are zoonotic bacteria that can cause severe diseases (anthrax, brucellosis, tularemia and plague, respectively) in humans and animals [1–4]. Because of their ease of dissemination and the potentially high mortality rate after inhalational exposure, *B. anthracis*, *F. tularensis* and *Y. pestis* are classified as category A bioterrorism agents according to the CDC. *Brucella* spp. are especially infectious in aerosolized form, and can survive for long periods in the environment. Therefore, they are classified as category B bioterrorism agents by the CDC.

Rapid and reliable detection is essential for timely initiation of medical post-exposure prophylactic measures in the event of intentional release of these bacteria. The possibility of field testing should augment this capability, as compared with more conventional analysis performed in stationary laboratories. On the other hand, competence in the sampling and identification procedures under field conditions requires thorough training.

The powder form of *B. anthracis* spores is a well-known and highly pathogenic substance, whereas *Bacillus thuringiensis* is almost harmless to humans, even though it belongs to the same *Bacillus cereus* group as *B. anthracis* and shares many biological traits with it [5]. Therefore, the use of non-infectious *B. thuringiensis* in a training assay as a substitute for *B. anthracis* spores is preferable with regard to safety of the trainees.

In this study, two platforms for real-time PCR detection of *B. anthracis*, *Brucella* spp., *F. tularensis* and *Y. pestis* were compared. Furthermore, an assay for field-training purposes was developed using *B. thuringiensis* from a commercial powder formula insecticide as template.

Materials and Methods

Strains of bacteria

The strains used for specificity and sensitivity comparisons of the *B. anthracis*, *F. tularensis* and *Y. pestis* assays [6] between the RAZOR (Idaho Technology Inc., Salt Lake City, UT, USA) and ABI 7300/7500 (Applied Biosystems, Foster City, CA, USA) instruments are listed in Table 1. Strains or batches of bacterial DNA used for the development of the *Brucella* spp. and *B. thuringiensis* assays are listed in Tables 2 and 3, respectively. For the development of the *B. thuringiensis* assay, purified DNA from an insecticidal product (TUREX 50WP, Certis, Columbia, MD, USA), containing 50% *B. thuringiensis kurstaki-aizaway* strain GC-91 (lot 010774 M08 04) was used, whereas in the field laboratory training assay, the sample was prepared from this powdery substance with a simplified procedure, as described below.

Purification of DNA

DNA from the *B. anthracis*, *F. tularensis*, *Y. pestis* and *B. thuringiensis* strains was purified using the automated MagNA Pure Compact instrument and the MagNA Pure Nucleic Acid Isolation Kit I combined with the MagNA Pure Bacterial Lysis Buffer and proteinase K treatment (Roche, Basel, Switzerland), according to the manufacturer's instructions. DNA from the clinical *Brucella* isolates was purified with the QiaAmp DNA miniKit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. The concentration and purity of the DNA batches were determined with the NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

Primer and probe selection

The primer and TaqMan minor groove binder probe sequences developed, as well as their target genes, are listed in Table 4. All new oligonucleotide primers and probes for this study were designed using Primer Express software

TABLE 1. Bacterial strains used and results of the specificity testing of the *Bacillus anthracis*, *Francisella tularensis* and *Yersinia pestis* real-time PCR assays on the RAZOR and ABI instruments

			Agent (target gene)							
			B. anthracis (*pag*)		*B. anthracis* (*cap*)		*F. tularensis* (*23 kDa*)		*Y. pestis* (*pla*)	
Species	Strain	Source[a]	ABI 7300/7500	RAZOR	ABI 7300/7500	RAZOR	ABI 7300/7500	RAZOR	ABI 7300/7500	RAZOR
Agrobacterium tumefaciens	C58C1/RP4	6	–	–	–	–	–	–	–	–
B. anthracis	ATCC 4229 (pXO1–/pXO2+)	4	–	–	+	+	–	–	–	–
B. anthracis	Sterne 7702 (pXO1+/pXO2–)	4	+	+	–	–	–	–	–	–
Bacillus cereus	ELMI 21	4	–	–	–	–	–	–	–	–
Bacillus licheniformis	ELMI 325	4	–	–	–	–	–	–	–	–
Bacillus mycoides	ELMI 44	4	–	–	–	–	–	–	–	–
Bacillus thuringiensis	ELMI 123	4	–	–	–	–	–	–	–	–
B. thuringiensis	ssp. *kurstaki-aizaway*	7	–	–	–	–	–	–	–	–
Brucella melitensis	72, biotype 3	2	–	–	–	–	–	–	–	–
Burkholderia multivorans	DSM 13243	8	–	–	–	–	–	–	–	–
Enterobacter cloacae	tks461	6	–	–	–	–	–	–	–	–
Escherichia coli	C600/pYET6	6	–	–	–	–	–	–	–	–
Francisella philomiragia	DSM 7535	8	–	–	–	–	–	–	–	–
F. tularensis	LVS (ATCC 29684)	5	–	–	–	–	+	+	–	–
Microbacterium barkeri	DSM 20145	8	–	–	–	–	–	–	–	–
Moraxella catarrhalis	035E	6	–	–	–	–	–	–	–	–
Staphylococcus aureus	ATCC 25923	6	–	–	–	–	–	–	–	–
Yersinia bercovieri	3016/84	6	–	–	–	–	–	–	–	–
Yersinia enterocolitica	1309/80	6	–	–	–	–	–	–	–	–
Yersinia frederiksenii	38/83	6	–	–	–	–	–	–	–	–
Yersinia intermedia	9/85	6	–	–	–	–	–	–	–	–
Yersinia kristensenii	119/84	6	–	–	–	–	–	–	–	–
Yersinia mollaretii	92/84	6	–	–	–	–	–	–	–	–
Y. pestis	EV76-c	6	–	–	–	–	–	–	+	+
Y. pestis	KIM D1	6	–	–	–	–	–	–	+	+
Yersinia pseudotuberculosis	H305-36/89	6	–	–	–	–	–	–	–	–
Y. pseudotuberculosis	No. 90	6	–	–	–	–	–	–	–	–
Y. ruckeri	RS41	6	–	–	–	–	–	–	–	–

[a]The sources are as follows: 1, Department of Applied Chemistry and Microbiology, Faculty of Agriculture and Forestry, University of Helsinki, Finland; 2, Institute of Microbiology, German Armed Forces, Munich, Germany; 3, Turku University Central Hospital and Helsinki District Central Hospital Laboratory Diagnostics HUSLAB; 4, Laboratory Strain Collection, Finnish Food Safety Authority Evira; 5, Laboratory Strain Collection, FOI, Umeå, Sweden; 6, Laboratory Strain Collection, Department of Bacteriology and Immunology, University of Helsinki; 7, Purified from a commercial insecticide; 8, DSMZ, German Collection of Microorganism and Cell Cultures, Braunschweig, Germany.

TABLE 2. Bacterial strains used for the specificity testing of the *Brucella* spp. (IS*711*)-specific PCR assay

Species	Strain	Source[a]	Results	
			ABI 7300/7500	RAZOR
Agrobacterium rhizogenes	HAMBI 1816 T	1	–	–
Agrobacterium tumefaciens	HAMBI 1811 T	1	–	–
Agrobacterium vitis	HAMBI 1817 T	1	–	–
Bacillus anthracis	ATCC 4229	4	–	–
B. anthracis	Sterne 7702	4	–	–
Bacillus thuringiensis	ssp. *kurstaki-aizaway*	7	–	–
Bradyrhizobium japonicum	HAMBI 2314 T	1	–	–
Brucella spp.	Eight clinical isolates	3	+	+
Brucella abortus	7, biotype 6	2	+	+
Brucella abortus	91, biotype 1	2	+	+
Brucella abortus	101, biotype 3	2	+	+
Brucella abortus	120, biotype 4	2	+	+
Brucella abortus	86/8/59 Weybridge, biotype 2	2	+	+
Brucella abortus	NCTC 10093, biotype 1	2	+	+
Brucella abortus	NCTC 10501, biotype 2	2	+	+
Brucella abortus	NCTC 10502, biotype 3	2	+	+
Brucella abortus	NCTC 10503, biotype 4	2	+	+
Brucella abortus	NCTC 10504, biotype 5	2	+	+
Brucella abortus	NCTC 10505, biotype 6	2	+	+
Brucella abortus	NCTC 10506, biotype 7	2	+	+
Brucella abortus	NCTC 10507, biotype 9	2	+	+
Brucella melitensis	1, biotype 2	2	+	+
Brucella melitensis	11, biotype 1	2	+	+
Brucella melitensis	72, biotype 3	2	+	+
Brucella melitensis	16M_NCTC_10094, biotype 1	2	+	+
Brucella melitensis	NCTC 10508, biotype 2	2	+	+
Brucella melitensis	NCTC 10509, biotype 3	2	+	+
Brucella suis	9, biotype 2	2	+	+
Brucella suis	9/2002, biotype 1	2	+	+
Brucella suis	NCTC 10510, biotype 2	2	+	+
Brucella suis	NCTC 10511, biotype 3	2	+	+
Brucella canis	125, biotype 1	2	+	+
Brucella canis	RM_6/66_ATCC_23365, biotype 1	2	+	+
Brucella neotomae	NCTC 10084 SK33	2	+	+
Brucella maris	2410	2	+	+
Brucella maris	2411	2	+	+
Brucella maris	NCTC 12890	2	+	+
Brucella maris	NCTC 12891	2	+	+
Burkholderia pseudomallei	HAMBI 33	1	–	–
Francisella tularensis	LVS (ATCC 29684)	5	–	–
Mesorhizobium huakuii	HAMBI 2035 T	1	–	–
Mesorhizobium loti	HAMBI 1129 T	1	–	–
Ochrobactrum anthropi	HAMBI 2402	1	–	–
Rhizobium calligum	HAMBI 2326 T	1	–	–
Rhizobium galegae	HAMBI 540 T	1	–	–
Rhizobium leguminosarum	HAMBI 14 T	1	–	–
Sinorhizobium meliloti	HAMBI 2148 T	1	–	–
Yersinia pestis	EV76-c	6	–	–
Y. pestis	KIM D1	6	–	–

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(ABI, Warrington, UK) versions 2.0 and 3.0, and synthesized by ABI. The primers and probes were selected on the basis of minimum dimer or secondary structure formation and minimum homology with other known prokaryotic gene sequences.

Out of several primer pairs and probes initially designed and tested for the genus-specific *Brucella* spp. assay, those selected were based on the *Brucella melitensis* strain 16M insertion sequence IS*711* (GenBank accession number NC003317.1, derived from reference sequence AE008917), which is highly conserved in the genus *Brucella*, with varying locations and copy numbers in each species [7–9]. This target has been used previously in *Brucella* species-specific real-time PCR assays [10,11].

The ability to produce crystal toxins, encoded by mainly plasmid-located crystalline genes (*cry* genes), is mostly responsible for the specific insecticidal activity of *B. thuringiensis* species, and accounts for the use of these microbes as insecticides [12–14]. The *cry1Ac* gene (accession number U87397) of *B. thuringiensis* ssp. *kurstaki*, a bacterium present in the commercial insecticidal powder formula, was used as a target in the developed training assay for *Bacillus* spore detection. The gene fragment predicted to be produced by the designed primers on the basis of the *B. thuringiensis* ssp.

TABLE 3. Specificity panel for and results of the assay for *Bacillus thuringiensis*

Species	Strain	Source[a]	Agent (target gene) *B. thuringiensis* (*cry*)[b] ABI 7300/7500	RAZOR
Bacillus anthracis	ATCC 4229 (pXO1−/pXO2+)	3	−	−
B. anthracis	Sterne 7702 (pXO1+/pXO2−)	3	−	−
Bacillus cereus	ELMI 21	3	−	−
Bacillus circulans	HAMBI 474	1	−	−
Bacillus coagulans	HAMBI 385	1	−	−
Bacillus licheniformis	ELMI 325	3	−	−
Bacillus mycoides	ELMI 44	3	−	−
Bacillus subtilis	HAMBI 251	1	−	−
B. thuringiensis	ELMI 123	3	+	+
B. thuringiensis	ssp. *kurstaki-aizaway*	6	+	+
Brucella melitensis	72, biotype 3	2	−	−
Francisella tularensis	LVS (ATCC 29684)	4	−	−
Yersinia pestis	EV76-c	5	−	−
Y. pestis	KIM D1	5	−	−

[a]The sources are as follows: 1, Department of Applied Chemistry and Microbiology, Faculty of Agriculture and Forestry, University of Helsinki, Finland; 2, Institute of Microbiology, German Armed Forces, Munich, Germany; 3, Laboratory Strain Collection, Finnish Food Safety Authority Evira; 4, Laboratory Strain Collection, FOI, Umeå, Sweden; 5, Laboratory Strain Collection, Department of Bacteriology and Immunology, University of Helsinki; 6, Purified from a commercial insecticide.
[b]The results were identical with both variants of the *B. thuringiensis* assay: the field test and the optimized test with higher sensitivity.

kurstaki sequence was not found in other microorganism sequences available in the GenBank database, except in *B. thuringiensis* when analysed by the BLAST algorithm.

PCR and sequencing equipment and procedures

Optimal primer concentrations for the *Brucella* spp. and *B. thuringiensis* assays, as well as for all RAZOR platform assays, were determined graphically from plotted reaction curves, using both the lowest crossing threshold (C_t) and the highest fluorescence signal as the criteria in a matrix-type evaluation. On the basis of re-evaluation of the primer and probe concentrations in the assays targeting the *B. anthracis cap* and *pag*, *F. tularensis 23 kDa* and *Y. pestis pla* genes on the ABI platform, no changes were made to those reported earlier [6].

All PCR assays on the ABI 7300 (software version 1.2.3) or ABI 7500 (software version 1.3.1) instruments were run in a final volume of 25 μL, consisting of 12.5 μL of 2 × master mix (ABI), 2.5 μL of purified DNA template, 2.5 μL of internal positive control (IPC) master mix and 0.5 μL of IPC

TABLE 4. Primers and probes for the developed real-time PCR assays for the ABI and RAZOR platforms

Target organism	Target gene	Primer and probe sequences	Final concentration (nM) ABI 7300/7500	RAZOR	Amplicon length (bp)	Reference
Brucella spp.	IS*711*	Forward 5′-GGC CTA CCG CTG CGA AT-3′	300	300	54	This study
		Reverse 5′-TTG CGG ACA GTC ACC ATA ATG-3′	900	300		
		Probe 5′-FAM-AAG CCA ACA CCC GGC-MGBNFQ-3′	250	250		
Bacillus thuringiensis	*cry*[a]	Forward 5′-GCT TCT CCT GTC GGT TTT TCG-3′	900	900	66	This study
		Reverse 5′-TGC ATT TCC CAT GGT TCC A-3′	900	900		
		Probe 5′-FAM-CCA GAA TTC ACG TTT CC-MGBNFQ-3′	250	250		
	cryT[b]	Forward 5′-ATG GCT TCT CCT GTA GGG TTT TC-3′	300	300	71	This study
		Reverse 5′-GCT GCA TTT CCC ATA GTT CCA-3′	900	900		
		Probe 5′-FAM-CCA GAA TTC ACT TTT CCG CT-MGBNFQ-3′	250	250		
Bacillus anthracis	*cap*	Forward 5′-TTG GGA ACG TGT GGA TGA TTT-3′	300	300	69	[6]
		Reverse 5′-TCA GGG CGG CAA TTC ATA AT-3′	900	900		
		Probe 5′-FAM-TAG TAA TCT AGC TCC AAT TGT-MGBNFQ-3′	250	250		
	pag	Forward 5′-CGG ATA GCG GCG GTT AAT C-3′	300	300	85	[6]
		Reverse 5′-CAA ATG CTA TTT TAA GGG CTT CTT TT-3′	900	900		
		Probe 5′-FAM-TAG AAA CGA CTA AAC CGG ATA T-MGBNFQ-3′	250	250		
Francisella tularensis	*23 kDa*	Forward 5′-TGA GAT GAT AAC AAG ACA ACA GGT AAC A-3′	300	900	84	[6]
		Reverse 5′-GGA TGA GAT CCT ATA CAT GCA GTA GGA -3′	900	900		
		Probe 5′-FAM-CCA TTC ATG TGA GAA CTG-MGBNFQ-3′	250	250		
Yersinia pestis	*pla*	Forward 5′-GAA AGG AGT GCG GGT AAT AGG TT-3′	50	300	63	[6]
		Reverse 5′-CCT GCA AGT CCA ATA TAT GGC ATA-3′	300	900		
		Probe 5′-FAM-TAA CCA GCG CTT TTC-MGBNFQ-3′	250	250		

[a]Field test; based on NBCI databank accession number U87397.
[b]Optimized assay based on the partially sequenced *cry* of the TUREX insecticide powder.

synthetic DNA, and appropriate volumes of primer and probe solutions to obtain the final concentrations presented in Table 4. After an initial 50°C incubation for 2 min for activation of UNG enzyme, and 95°C for 10 min for denaturation of template and activation of the polymerase enzyme, all assays on the ABI platforms were performed by repeating the following procedure 40 times: 95°C for 15 s, and 60°C for 1 min. With these thermocycling parameters, the PCR took approximately 100 min.

The RAZOR instrument (software version 1, beta 1; Idaho Technology) allows the use of only three different temperatures with three steps and a maximum of 4 min per step for thermocycling. The initial 10-min heating step used with the ABI platform was therefore not applicable. Two types of pouch were used with the RAZOR instrument: one type has four inlet ports for four samples, which are each drawn to three wells (4×3 pouches). The other type contains six inlet ports for six samples, and each sample is run in duplicate (6×2 pouches). The volume of the PCR reaction mixture for one syringe used for infusion of the 4×3 pouch was 500 μL, consisting of 250 μL of the master mix (Takara Premix Ex Taq; Takara, Shiga, Japan), 50 μL of template, and appropriate volumes of primer and probe solutions and DNase/RNase-free water to obtain the final concentrations presented in Table 4. For the 6×2 pouch, the syringe volume of the reaction mixture was 333 μL. Finally, however, the final reaction volume was 100 μL per well with both types of pouch.

The default thermocycling programme of the RAZOR instrument consisted of an initial denaturation step at 94°C for 2 min, followed by 55 cycles at 94°C for 10 s and 60°C for 30 s. The use of the Takara Premix Ex Taq polymerase allowed shortening of the initial denaturing phase to 10 s and the cycle denaturing phase at 94°C to 5 s. With these thermocycling parameters, the reaction took approximately 40 min to run, after which the results could be viewed on the screen of an attached computer.

The sensitivities (limits of detection) of the assays were estimated with either of the technically similar ABI (7300 or 7500) and the RAZOR system, using ten-fold serial dilutions of corresponding DNA as template.

To determine the partial DNA target sequence of the commercial *B. thuringiensis* ssp. *kurstaki-aizaway* preparation for further optimization of the used primer pair, cloned (TOPO TA cloning kit; Invitrogen Corporation, Carlsbad, CA, USA) PCR products obtained with newly designed outer PCR primers (data not shown) were analysed with an automated ABI 3130XL DNA sequencer (ABI), using Big Dye chemistry (ABI) and previously published procedures [15].

Trial with the *B. thuringiensis* training assay in the mobile field laboratory

The mobile field laboratory met BSL-3 safety level standards, and had been designed for the Finnish Defence Forces. It was equipped with a line of steel cabinets consisting of: a sample hatch, into which a sample can be safely transported from the outside; a class III microbial safety cabinet (MSC); a class II MSC; and an autoclave for safe disposal of wastes. The real-time PCR instrument was placed inside the field laboratory outside of the cabinets.

In order to estimate the number of viable *B. thuringiensis* spores to be used in the following trial, serial dilutions in phosphate-buffered saline (PBS) of a TUREX insecticide sample were cultivated in Tryptone Soy Agar medium. The total number of spores in the preparation was estimated in a microscopy counting chamber (Bürker-Türk, Marienfeld, Germany). Field-training sample material was prepared by mixing the TUREX powder thoroughly with rye flour of similar granularity and colour. The samples consisted of 0.5-g aliquots of the rye flour containing 10% or 1% TUREX in 50-mL sample tubes. Unspiked rye flour was used as negative control. Three coded training samples containing predetermined amounts of TUREX powder and rye flour were transported to a mobile field laboratory, where the sample processing and real-time PCR analyses with the training assay using the *cry* primers and probe took place. One negative sample containing PBS only was included in the sample processing to detect any carryover contaminants.

To obtain the template for PCR analysis during the trial, samples of the powder substance were suspended in PBS. After thorough mixing, 1 mL of the suspension was filtered through a 1.2-μm filter (Whatman GmbH, Dassel, Germany), and this was followed by a second filtration through a 0.2-μm filter (Whatman GmbH). The sample filtration was performed inside a class III MSC, and this was followed by pipetting of all PCR reagents and the templates into the reaction pouches inside a class II MSC before analysis with the RAZOR instrument.

Results

The analytical specificity results obtained with the assays for the agents of anthrax, tularemia and plague, as well as with the developed *Brucella* assay, are displayed in Tables 1 and 2. No unspecific cross-reactivity of the assays with other bacteria was observed in these experiments.

The sensitivities of the comparative PCR assays ranged from 10 to 100 fg of specific chromosomal target DNA per reaction, using either the ABI 7300/7500 platform

(0.4–4 ng/mL) or the RAZOR field instrument (0.1–1 ng/mL) (Table 5). However, no difference in performance between the two technology platforms (ABI and RAZOR) was observed when optimally purified target DNA was used as template in the assays for anthrax, brucellosis, tularemia and plague.

The analytical specificity results obtained with the use of closely related bacteria as template in the developed field-training assay for *B. thuringiensis* are shown in Table 3. No cross-reactivity with other *Bacillus* species was detected. The detection ranges of the field assay with purified TUREX insecticide as template were 250–25 ng of powder per reaction with the ABI instrument, and 50 μg to 1 ng per reaction with the RAZOR instrument. No amplification occurred at concentrations outside these ranges. To determine the cause of this notable difference in the detection range between the two platforms, we sequenced the partial *cry* target of the commercial product, and, indeed, the TUREX *B. thuringiensis* ssp. *kurstaki-aizaway* target sequence revealed minor mismatches in the targeted primer and probe sequence areas (two mismatches were found in the forward primer and one mismatch both in the reverse primer and in the probe) as compared with the original *B. thuringiensis* ssp. *kurstaki-aizaway* sequence used as the basis for primer and probe selection (GenBank accession number U87397).

After modification of the oligonucleotide sequences to make them identical to the corresponding sequence in TUREX powder, the sensitivity of the assays with this preparation as template increased considerably, and only a slight difference between the assay platforms continued to be observed, the detection ranges being 250 ng to 25 pg of powder per 25-μL reaction (10 μg/mL to 1 ng/mL) with the ABI system, and 50 μg to 100 pg per 100-μL reaction (500 μg/mL to 1 ng/mL) with the RAZOR instrument. This consistency in the wider range of analytical sensitivity suggests greater chemical robustness in the PCR process within the RAZOR platform. As with the originally designed assay, no cross-reactivity with other *Bacillus* strains was seen with the modified assay (Table 3; Fig. 1). When purified cloned target DNA from the TUREX preparation was used as template in the modified assay, no significant difference in assay sensitivity (100 ag per reaction) between the two platforms was detected (Table 5). With chromosomal DNA purified from bacterial isolates grown from the TUREX powder, the sensitivities of the assays were 1 ng per reaction and 100 fg per reaction on the ABI 7300/7500 platforms, and 100 fg per reaction and 100 fg per reaction with the RAZOR system, with *cry* (originally designed) and *cryT* (modified) assays, respectively, suggesting greater analytical flexibility of the RAZOR detection system with primers and a probe that contained mismatches (Table 5).

In a blindly performed field test with the RAZOR instrument in a mobile laboratory, analytical tests with two *B. thuringiensis*-spiked rye flour samples as well as a negative control sample containing only rye flour were determined correctly as positives and negative, respectively (Fig. 2).

TABLE 5. The sensitivities of the developed real-time PCR assays on the two different platforms

Target organism	Target gene	Sensitivity (limit of detection)[a]	
		ABI 7300/7500 instruments	RAZOR field instrument
Brucella spp.	IS*711*	10 fg	10 fg
Bacillus anthracis	*cap*	100 fg	100 fg
	pag	10 fg	10 fg
Francisella tularensis	*23 kDa*	10 fg	10 fg
Yersinia pestis	*pla*	10 fg	10 fg
Bacillus thuringiensis	*cryT*[b]	100 fg	100 fg
B. thuringiensis	*cryT*[c]	100 ag[c]	100 ag[c]

[a]Amount of genomic DNA as template.
[b]Optimized assay with modified oligonucleotides based on the partially sequenced *cry* of TUREX insecticide powder.
[c]Amount of cloned plasmid DNA as template.

Discussion

Sensitive and rapid real-time PCR detection methods have, especially in the aftermath of the anthrax letters of 2001, been developed and refined for the CDC list A agents, including the causative agents of anthrax [16,17], tularemia [18,19] and plague [20,21]. Diagnostics for the CDC list B agents, such as *Brucella* spp., the cause of brucellosis, have also been updated and further developed [11,22]. All of these reports focus on the identification of single or closely related pathogens, and mainly describe methodologies that are confined to stationary clinical diagnostic laboratories. However, there is a definite need for a broader range of applications for detection and identification of a panel of selected agents under field conditions, to be used, for example, by the veterinary public health service. All four agents singled out above are zoonotic, and the first indication of their intentional release might well arise as an animal disease outbreak. There are only a few earlier reports describing features of commercial hand-held or portable instruments for rapid diagnosis of bioterrorism agents [23–26], but there seem to be no previous scientific reports on actual field testing of such devices.

Notable differences in analytical sensitivity or specificity performance between the ABI and RAZOR platforms were not observed in our study when optimally purified template DNA and target-specific oligonucleotides were used. Similar

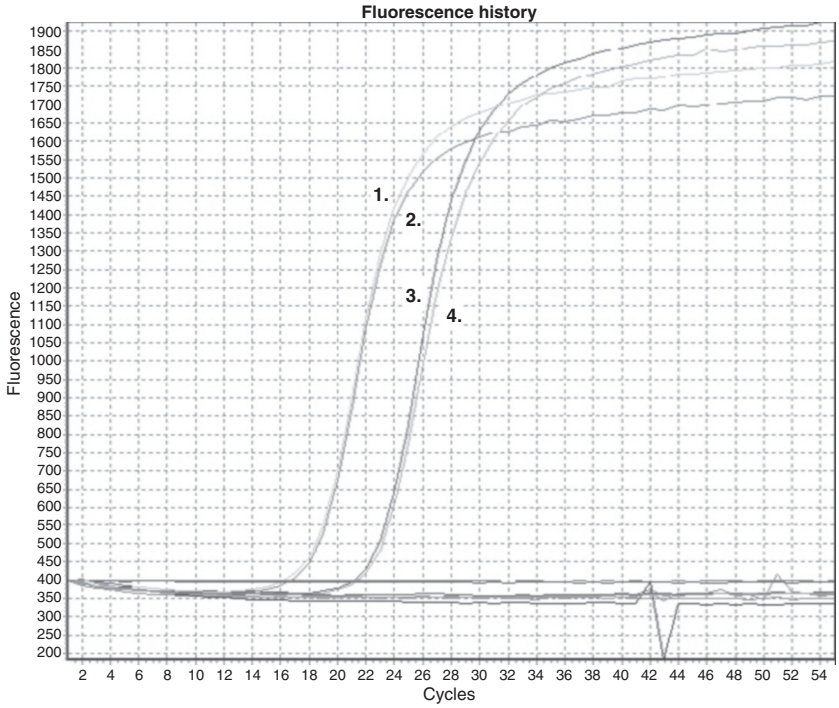

FIG. 1. A representative *Bacillus thuringiensis* real-time PCR amplification plot obtained with the RAZOR instrument to test the specificity of the optimized primers (target gene *cryT*). Four different *Bacillus* strains (ELMI 21, ELMI 44, ELMI 123 and ELMI 325) were analysed. As the positive control, 400 pg of chromosomal DNA from the TUREX insecticide powder was used per reaction. No template controls (NTCs) were included as negative controls. All assays were performed in duplicate, using the 6 × 2 pouches. Both the positive control (amplification curves 1 and 2) and the *B. thuringiensis* strain (ELMI 123; amplification curves 3 and 4) showed amplification, the average C_t values being 17.1 and 21.6, respectively. The other *Bacillus* strains tested (ELMI 21, ELMI 44 and ELMI 325), as well as the NTCs, were negative.

findings have been recently reported by Christensen *et al.* [27]. They compared assay performance in detecting biological threat agents with the RAPID, LightCycler and the Smart Cycler platforms under optimal laboratory conditions. The analytical limits of detection in that report varied between 50 and 100 fg for most assays [27], which is in concordance with our results. However, implementation of these methodologies in field conditions was not performed as in our study. We have successfully utilized the ABI 7300 instrument in a mobile laboratory setting [26], and in this current article we describe the development of a field-training assay for *Bacillus* spore detection that was evaluated under field conditions with the RAZOR instrument. During the development of this field assay, we observed a slight increase in assay robustness with the RAZOR instrument when using oligonucleotides that contained mismatches with the target sequence, as well as when suboptimally prepared template was analysed. The demonstrated difference in the detection range of the instrument platforms may be partially explained by the larger reaction volume (100 μL) in the RAZOR instrument than in the ABI instrument (25 μL). This larger reaction volume may contribute to resistance against inhibitors of the PCR. On the other hand, we did not observe any cross-reactivity deriving from unspecific oligonuclotide hybridization with either of the platforms.

The ABI 7300 instrument is heavy (29 kg) and requires more space (width × depth × height = 34 × 45 × 49 cm), but it is versatile, and the tungsten–halogen lamp technology has, in our hands, proven to be robust enough for mobile laboratory conditions [26]. Conversely, the RAZOR instrument was originally designed for field use, and because it is smaller and lighter (width × depth × height = 17 × 11 × 23 cm, weight = 4.1 kg), it can be easily transferred by carrying. However, both the ABI thermocycler and the RAZOR instrument are currently dependent on a field laboratory or similar setting for sample processing and DNA purification. Furthermore, there are significant differences in the time needed for thermocycling between the two platforms, i.e. 40 min and 100 min for the RAZOR and the ABI platform, respectively.

The development of the assays that were used for comparison of these two assay platforms for the agents of anthrax, tularemia and plague has been published previously

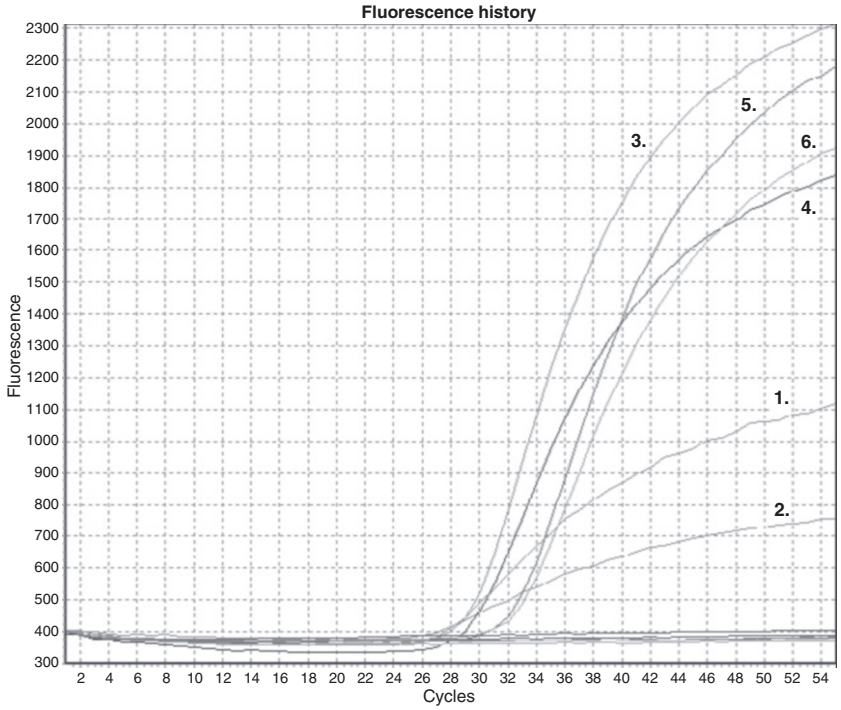

FIG. 2. Analytical test results from the field trial with the RAZOR instrument. Positive controls (amplification curves 1 and 2) and *Bacillus thuringiesis*-positive samples (amplification curves 3 and 4, containing 0.05 g of TUREX insecticide combined with 0.5 g of rye flour, and amplification curves 5 and 6, containing 0.005 g of TUREX insecticide combined with 0.5 g of rye flour) showed the expected positive fluorescence signals. Neither the negative samples nor the non-template control showed any PCR reactivity.

[6]. The gene target for the newly developed *Brucella* spp. assay was selected on the basis of the findings of Redkar *et al.* [10] regarding their development of the *Brucella* species-specific assays. However, recently, Al Dahouk *et al.* [28] evaluated *Brucella* spp. genus-specific and species-specific real-time PCR assays, and found that not all strains of *Brucella abortus* or *Brucella suis* were detected by the assay developed by Redkar *et al.* [10]. Therefore, for our study, primers and probes targeting IS*711* were newly designed to detect all species of the genus *Brucella* for rapid broad-range detection preceding confirmatory species-specific detection and identification in a stationary laboratory. In fact, recently, an assay for *Brucella* spp. using similar oligonucleotide targets on IS*711* as well as multiple assays specific for different *Brucella* species have been developed [29].

The sensitivity of our *Brucella* spp. assay proved to be slightly greater (10 fg per reaction) than that reported by Al Dahouk *et al.* in 2007 for their *bcsp31* assay (16–18 fg per reaction). Furthermore, in a recently published comparative study by Bounaadja *et al.* on the target genes for *Brucella* spp. identification, the authors concluded that IS*711* was a more sensitive target than *bcsp31* and *per*. However, in their study, the sensitivity varied between different *Brucella* species, owing to different copy numbers of the IS*711* sequence in each species [30].

The plasmid-encoded *cry* genes constitute a specific feature of the different *B. thuringiensis* species [14] in the otherwise genetically quite homologous *B. cereus* group, and are therefore useful in specific assays for the differentiation of *B. thuringiensis* from other *Bacillus* species. The simplified sample preparation procedure described in this article for the surrogate *B. anthracis* TUREX powder bypasses the need for sensitive biochemical materials or equipment, such as specific enzymes or centrifuges, before PCR amplification of the analyte. Of note is that, the pretreatment procedure applies, as such, only to the concentrated insecticide spore preparation (TUREX) used in this study. Both the spore plate count and the direct estimation of the number of spores with the counting chamber indicated a level of approximately 2×10^{10} spores/g of TUREX powder. In our hands, the described field-training assay, starting with the devised simplified preparation of the sample and using the RAZOR instrument, is straightforward and safe. Therefore, it is adequate for field-training purposes, e.g. for analyses of powder letters presumably containing anthrax spores.

These assays provide rapid tools for the specific detection of four biological threat agents. Although no notable differences between the ABI 7300/7500 and RAZOR instruments were observed in analytical sensitivity or specificity under optimal conditions, the duration of thermocycling with the RAZOR instrument was significantly shorter (40 min vs. 100 min with ABI 7300/7500). Furthermore, the physical robustness and smaller size of the RAZOR apparatus adds to its usefulness under field conditions. Moreover, our

results suggest that the RAZOR chemistry may be more resistant to suboptimal PCR conditions. However, integrated or simplified sample preparation procedures, as described here for *Bacillus* spore detection, should be further developed.

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Transparency Declaration

The authors have no dual or conflicting interests.

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